



TITLE:

Small RNAs detected in exosomes derived from the MH7A synovial fibroblast cell line with TNF- α stimulation

AUTHOR(S):

Takamura, Yosuke; Aoki, Wataru; Satomura, Atsushi; Shibasaki, Seiji; Ueda, Mitsuyoshi

CITATION:

Takamura, Yosuke ...[et al]. Small RNAs detected in exosomes derived from the MH7A synovial fibroblast cell line with TNF- α stimulation. PLOS ONE 2018, 13(8): e0201851.

ISSUE DATE:

2018-08-10

URL:

<http://hdl.handle.net/2433/234096>

RIGHT:

© 2018 Takamura et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

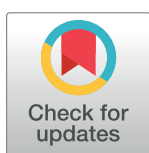
RESEARCH ARTICLE

Small RNAs detected in exosomes derived from the MH7A synovial fibroblast cell line with TNF- α stimulation

Yosuke Takamura¹, Wataru Aoki¹, Atsushi Satomura¹, Seiji Shibasaki², Mitsuyoshi Ueda^{1*}

1 Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kitashirakawa Oiwake-cho, Sakyo-ku, Kyoto, Japan, **2** General Education Center, Hyogo University of Health Sciences, Kobe, Japan

* miueda@kais.kyoto-u.ac.jp



OPEN ACCESS

Citation: Takamura Y, Aoki W, Satomura A, Shibasaki S, Ueda M (2018) Small RNAs detected in exosomes derived from the MH7A synovial fibroblast cell line with TNF- α stimulation. PLoS ONE 13(8): e0201851. <https://doi.org/10.1371/journal.pone.0201851>

Editor: Yun Zheng, Kunming University of Science and Technology, CHINA

Received: April 17, 2018

Accepted: July 22, 2018

Published: August 10, 2018

Copyright: © 2018 Takamura et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: The raw sequencing data are available from the database of the National Center for Biotechnology Information (SRA accession: SRP136572).

Funding: This work was supported by JST CREST (Grant No. JPMJCR16G2, <http://www.jst.go.jp/kisoken/crest/en/index.html>) (MU). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Rheumatoid arthritis (RA) is an autoimmune disease that causes the chronic inflammation of the joints. Intercellular communication containing synovial fibroblasts seems to play a major role in RA pathogenesis. In this study, to better understand intercellular communication related to RA pathogenesis, we identified exosomal microRNAs (miRNAs) derived from synovial fibroblasts. Exosomes were collected from an RA synovial fibroblast (RASf) cell line, namely, MH7A, with or without stimulation by tumor necrosis factor alpha (TNF- α). We used small RNA sequencing to analyze the profile of small RNAs, including miRNAs, in MH7A exosomes and cells. By using differential expression analysis, we identified four miRNAs (miR-155-5p, miR-146a-5p, miR-323a-5p, and miR-1307-3p) that are upregulated in exosomes with TNF- α stimulation. The identification of miR-155-5p and miR-146a-5p which have been reported in RA patients demonstrated the validity of our experimental model. Other two miRNAs were newly identified. miR-323a-5p was predicted to target the protein encoding gene *CD6*, which attenuates T-cell activation signals, and miR-1307-3p was predicted to target the protein encoding gene N-myc downstream-regulated gene 2 (*NDRG2*), which inhibits osteoclast-related gene expression. The results suggested that these miRNAs might be involved in RA pathogenesis. We hope our results will help us understand the role of RASf exosomes in RA pathogenesis.

Introduction

Rheumatoid arthritis (RA) is a well-known autoimmune disease characterized by chronic synovial inflammation leading to joint destruction [1]. The RA synovium comprises abnormally proliferating synovial fibroblasts, macrophages, and infiltrated leucocytes [2]. RA synovial fibroblasts (RASfs) play a major role in the formation of intercellular communication networks in the synovium via chemokines and inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) and interleukin-6 (IL-6), which drive synovial inflammation and joint

Competing interests: The authors have declared that no competing interests exist.

destruction [2–4]. However, despite advances in RA treatment with biological agents, there are still some cases of uncontrolled synovial inflammation [5].

Exosomes, a type of extracellular vesicle, also participate in intercellular communication; they play a role in transporting bioactive molecules such as proteins and various types of RNAs, including microRNAs (miRNAs) [6]. Exosomes are secreted from all types of cells and are present in all biological fluids [6]. Exosomal miRNAs show physiological and pathological functions including immune regulation and cancer progression [7, 8], and the exosome transportation is not just a dead cell clearance mechanism [9]. For example, Ochiya's group showed that breast cancer-derived exosomes containing miR-181c trigger the breakdown of blood-brain barrier, and lead to brain metastasis [10]. There are several pathways in exosome uptake: receptor-ligand interactions, direct membrane fusion, and endocytosis/phagocytosis [11]. Studies showed that the primary pathway is internalization via micropinocytosis, clathrin-mediated and caveolin-mediated endocytosis, and phagocytosis. Many exosomes are targeted to the lysosome, but some of them escape lysosomal degradation via back fusion and release their contents into the cytoplasm [12]. Released miRNAs show their function in various ways [13]. For example, some portion of exosomal miRNAs associate with AGO2 and show the classic role of targeting mRNAs with the RISC components [14]. Furthermore exosomal miRNAs have entirely new functions as ligands for activation of immune cells via interaction with toll-like receptors [15]. In RA, exosomes derived from the synovial fluid contain citrullinated proteins [16], and RASF-derived exosomes contain a membrane form of TNF- α [17]. The molecular cargo in exosomes changes according to the origin and state of the cells, thus suggesting that exosomal proteins are involved in RA pathogenesis and that exosomes have the potential to be used as biomarkers [6, 7].

miRNAs are regulatory, noncoding RNA molecules comprising ~22 nucleotides. According to miRBase (<http://www.mirbase.org/>), an miRNA sequence and annotation database, more than 1800 miRNAs have been discovered in the human genome [18]. miRNAs play an important role in biological processes, including immune system regulation via interaction with partially complement messenger RNA (mRNA) to suppress mRNA translation [19, 20]. Several studies have evaluated miRNA expression in plasma, synovial tissue, and synovial fibroblasts in RA patients [21]. One study has shown that because miR-132 levels in the plasma of RA patients are different from those in healthy controls, miR-132 levels can be used as a potential biomarker [22]. Another study has shown that miR-155 and miR-146a levels increase in RASF [23]. Furthermore, miR-155-deficient mice do not develop collagen-induced arthritis (an RA model in mice), thus suggesting that miR-155 is involved in the chronic inflammation and joint destruction mechanisms [24]. Taken together, these miRNAs could contribute to RA pathogenesis and could be used as biomarkers for RA.

In addition to miRNAs, exosomes contain other types of noncoding RNAs, including ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), long noncoding RNAs (lncRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), Y RNAs, and vault RNAs (vtRNAs) [25–30]. These RNAs are different in function, localization, length, and structure. Although many questions still remain about these noncoding RNAs, their functions have become increasingly clear [25].

In this study, we focused on RASF-derived exosomal RNAs to elucidate their role in RA pathogenesis. We performed small RNA sequencing (RNA-Seq) by using Illumina MiSeq to analyze the miRNA expression of RASF exosomes. To construct a model of RA *in vitro*, we cultured MH7A cells—a human synovial fibroblast cell line derived from intra-articular soft tissues of the knee joints of RA patients [31]. The cells were stimulated with TNF- α to produce inflammation, and the profile of the exosomal miRNA was compared with that of unstimulated MH7A cells. Furthermore, we analyzed cellular RNA profiles to compare the expression

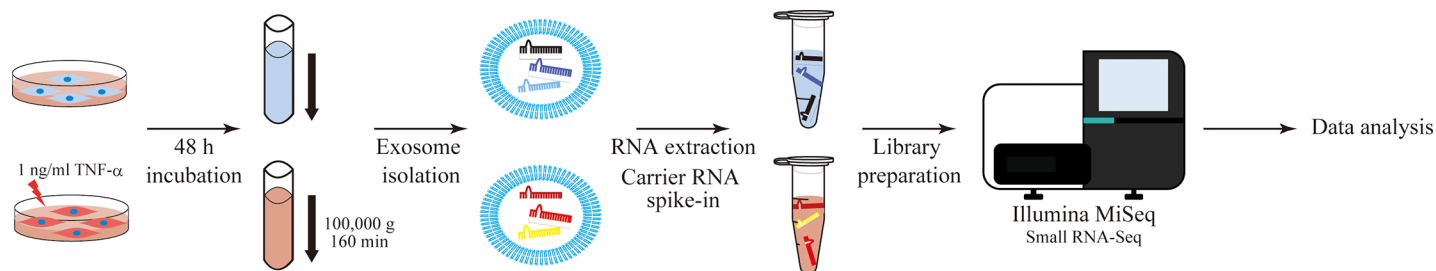


Fig 1. Experimental procedure of this study. Exosomes were isolated by ultracentrifugation from MH7A-conditioned media with or without TNF- α stimulation. Carrier RNA was used in the extraction step of exosomal RNAs. The extracted exosomal RNAs were sequenced on Illumina MiSeq.

<https://doi.org/10.1371/journal.pone.0201851.g001>

profiles between exosomes and cells. Fig 1 illustrates the experimental system of this study. We isolated MH7A exosomes from MH7A-conditioned media and quantified exosomal small RNAs. With TNF- α stimulation, four miRNAs were found to be upregulated in RASF exosomes. We believe our observations on RASF exosomal RNAs will help us understand the role of RASF exosomes in RA pathogenesis.

Materials and methods

Cell culture

In this study, we used the human synovial fibroblast cell line MH7A (Riken, Saitama, Japan) [4, 31]. MH7A cells were grown in a Biocoat collagen I 100 mm dish (Corning, NY, USA) with Roswell Park Memorial Institute 1640 Medium (Wako, Osaka, Japan). The medium was supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, MA, USA) and 1% antibiotic-antimycotic (Thermo Fisher Scientific). The culture plates were incubated at 37°C in a humidified atmosphere with 5% CO₂. For TNF- α stimulation and exosome isolation, 50% confluent cell dishes were washed twice with 5 mL phosphate-buffered saline (PBS) (Nacal tesque, Kyoto, Japan) and then incubated for 48 h in a medium supplemented with exosome-depleted FBS (System Biosciences, CA, USA) and with an antibiotic-antimycotic in the presence or absence of 1 ng/mL recombinant human TNF- α (Peprotech, NJ, USA).

IL-6 quantification using ELISA

We quantified the amount of IL-6 secreted from MH7A cells by using Quantikine enzyme-linked immunosorbent assay (ELISA) human IL-6 (R&D Systems, MN, USA) according to the manufacturer's instructions. Three independent experiments were performed. The cells remaining in each dish were treated with trypsin (Nacal tesque) and counted thrice by using a hemocytometer (Waken B Tech, Kyoto, Japan). The amount of IL-6 was measured twice by using a VMax® Kinetic Microplate Reader (Molecular Devices, CA, USA) set to 490 nm with 564 nm wavelength correction. The standard curve was fitted with a four-parameter logistic curve by using ImageJ [32], and the results were then calculated. Statistical significance was determined by Welch's t-test.

Exosome isolation from MH7A-conditioned media

We isolated exosomes by ultracentrifugation from 200 mL of MH7A-conditioned media with or without TNF- α treatment, as described previously [33]. The conditioned media were collected and centrifuged at 2000 \times g for 10 min at 4°C to eliminate dead cells, followed by filtration of the supernatant through a 0.2 μ m filter unit (Thermo Fisher Scientific) to eliminate cell

debris. The filtrates were stored at 4 °C until ultracentrifugation. Exosomes were pelleted by ultracentrifugation at 100,000 × *g* for 160 min at 4 °C (SRP28SA1, Hitachi Koki, Tokyo, Japan). The exosome pellets were washed once by resuspending them in PBS and ultracentrifugation. The pellets were again resuspended in 50 μL of PBS, rapidly frozen in liquid nitrogen, and stored at −80 °C until use.

Preparation of cellular protein

The MH7A-conditioned media were collected, and the cells were washed twice with 5 mL of ice-cold PBS, followed by the addition of 1 mL of radioimmunoprecipitation assay buffer (Wako) supplemented with 10 μL of a protease inhibitor cocktail (Sigma Aldrich, MO, USA). The cells were scraped off by a cell scraper and transferred to a microtube. The cell suspensions were kept on ice for 30 min with a vortex every 10 min. Cell debris was eliminated by centrifugation at 14,000 × *g* for 15 min at 4 °C, and the supernatant was collected.

Detection of protein by Western blot analysis

To quantify the protein concentrations of exosomes and cellular lysates, we used bicinchoninic acid assay (Protein Assay Bicinchoninate Kit, Nacalai tesque) according to the manufacturer's instructions. In a reducing condition for the detection of HSP70 [34], cellular lysates and exosome samples (1.5–2.0 μg) were mixed with a sample buffer solution with 2-mercaptoethanol for sodium dodecyl sulfate polyacrylamide gel electrophoresis (Nacalai tesque) and then heated for 3 min at 98 °C. In a nonreducing condition for the detection of CD63 and CD81 [34], cellular lysates and exosome samples were mixed with a sample buffer solution without 2-mercaptoethanol (Nacalai tesque) and incubated for 30 min at 37 °C. The samples were then electrophoresed on in-house 15% polyacrylamide gels and blotted on nitrocellulose membranes (Bio-Rad, CA, USA). The membranes were blocked for 1 h in Tris-buffered saline with 5% skim milk (Wako) and 0.05% Tween-20 (Nacalai tesque). Thereafter, they were incubated with monoclonal antibodies against CD63 (#Ts63, Thermo Fisher Scientific), CD81 (#1.3.3.22, Santa Cruz Biotechnology, CA, USA), or HSP70 (#MAB1663, R&D Systems); horseradish peroxidase-conjugated anti-mouse antibody (Bio-Rad) was used as a secondary antibody. Immunoreactive bands were detected using Chemi-Lumi One Super (Nacalai tesque) and Image Quant LAS 4000 mini (GE Healthcare, IL, USA).

RNA extraction

Exosomal RNAs and total cellular RNAs were isolated using the miRNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. First, carrier RNA was synthesized using Greiner Bio-One (Kremsmünster, Austria) (sequence: MS2 bacteriophage RNA [961–1000 bases] 5′-UCGCGACGUAUCGUGAUAUGGUUUUACAUAACGAUGCAC-3′). The carrier RNA sequence was confirmed to not match the human genome by using BLAST [35]. To isolate exosomal RNAs, 1 μg of carrier RNA was spiked-in lysates prepared by the QIAzol lysis reagent from the miRNeasy mini kit. The quantity, size, and purity of the isolated RNA samples were analyzed by using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) with Agilent RNA 6000 Pico Kit (Agilent Technologies) according to the manufacturer's instructions. The cellular RNAs of RNA integrity number > 7 were used for library preparation and are described in the following.

Library preparation and sequencing

cDNA libraries were prepared using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Set 1) (New England Biolabs, MA, USA) according to the manufacturer's instructions with modifications on library size selection. Amplified cDNA libraries were electrophoresed on 12.5% polyacrylamide gel (E-T 12.5L, ATTO, Tokyo, Japan), and ~140 nucleotide bands (the length of adapter-ligated miRNA constructs) were eluted and precipitated by ethanol. The library sizes were checked by a bioanalyzer using the Agilent High Sensitivity DNA Kit (Agilent Technologies). The libraries were quantified by using a 7500 real-time polymerase chain reaction system (Applied Biosystems, CA, USA) with KAPA Library Quantification Kits (Kapa Biosystems, MA, USA) according to the manufacturer's instructions. Furthermore, the libraries were equimolarly pooled for sequencing. Thereafter, sequencing was performed using MiSeq (Illumina, CA, USA) with single-end 36 cycles and 10% PhiX control (Illumina). The raw sequencing data are available from the database of the National Center for Biotechnology Information (SRA accession: SRP136572).

Data analysis

Fig 2 shows a flowchart of the data analysis conducted in this study. First, we assessed the quality of raw sequencing data by using FastQC (version 0.10.1 downloaded from <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Thereafter, we preprocessed raw sequence

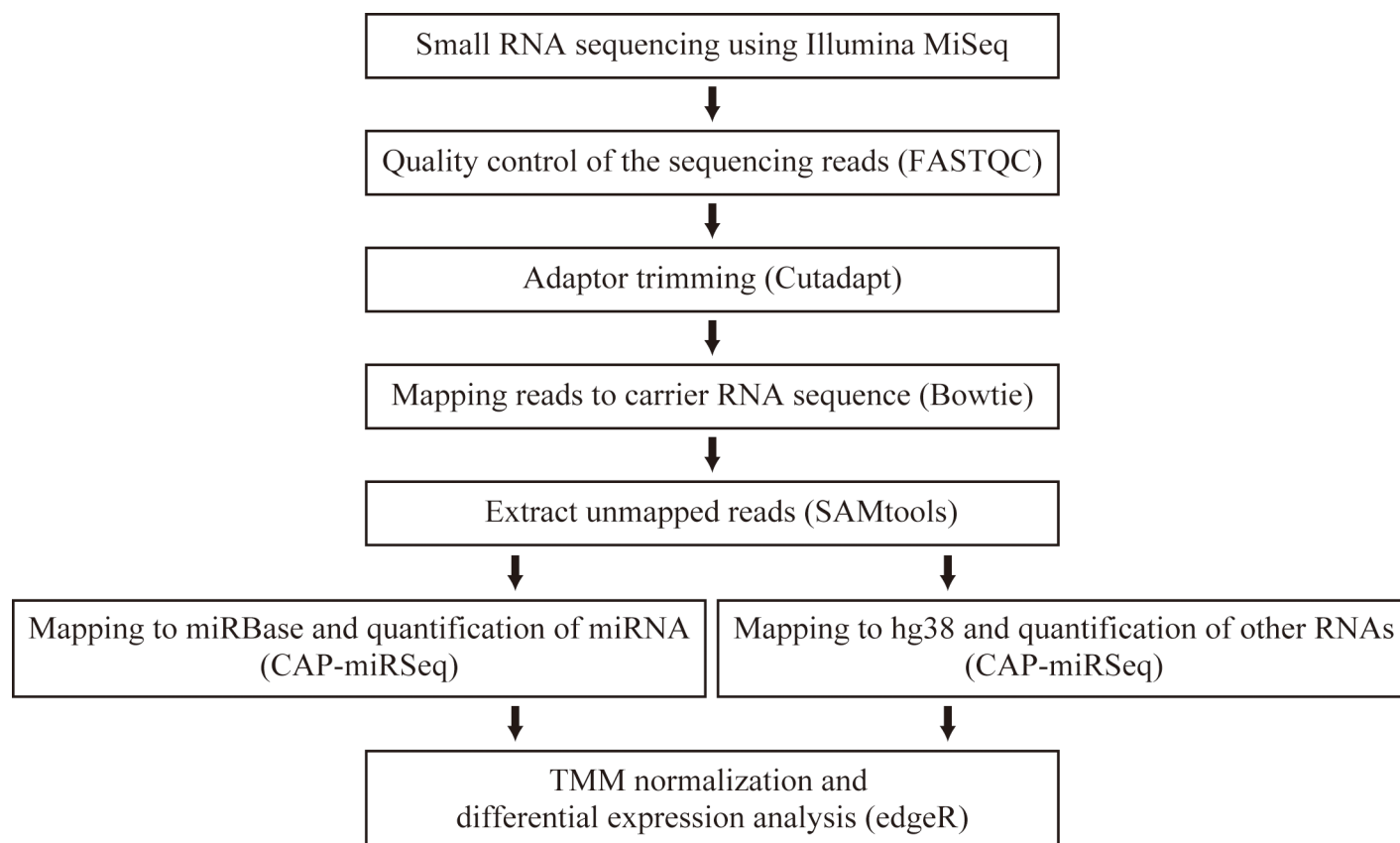


Fig 2. Flowchart of data analysis. After a quality check and adaptor trimming, the elimination of carrier RNA-Seq reads was performed using these bioinformatics tools. Thereafter, CAP-miRSeq was used to perform mapping and read counts. Differential expression analysis was conducted with edgeR. The software programs used for analysis are shown in parentheses.

<https://doi.org/10.1371/journal.pone.0201851.g002>

reads by using cutadapt (version 0.9.5) [36] to trim the adaptor sequences and remove reads that were too short (< 17 base pairs in length). To remove carrier RNA sequence reads from exosome libraries, adapter-trimmed reads were aligned to the carrier RNA sequence by using Bowtie (version 0.12.7) [37]. Unmapped to the carrier RNA sequences were extracted from the SAM file created and were converted to FASTQ format by using SAMtools (version 0.1.19) [38]. We then performed mapping against human genome GRCh38/hg38 and a known miRNA database (miRBase release 21) [18] by using CAP-miRSEQ (version 1.1) [39] with default parameters, which output the read counts of RNA categories in Ensembl 83 [40] and mature miRNA expression counts. The read counts of each RNA category were normalized with the number of reads per genome-aligned reads, and the average total number of each RNA category was calculated. The trimmed mean of M-values (TMM) normalization [41] and differential expression analysis were performed using an R package called edgeR (version 3.16.5) [42, 43] via the quantile-adjusted conditional maximum likelihood method [44]. For TMM normalization, lowly expressed transcripts (showing < 5 counts in four or more libraries) were filtered out. The differential expression of transcripts other than miRNAs was performed using genome-aligned reads without miRNA-aligned counts. Transcripts with a false discovery rate (FDR) below a threshold of 0.05 determined by edgeR were considered differentially expressed.

Target prediction of miRNAs

The target prediction of miRNAs was performed using DIANA-microT-CDS [45, 46] and TargetScan [47]. The DIANA-microT-CDS threshold was set to 0.7 (default). The result obtained using DIANA-microT-CDS included information about whether TargetScan also predicted the same genes. Only genes that were predicted by both algorithms were retrieved.

Results

Stimulation of MH7A cells by TNF- α

We cultured MH7A cells obtained from RA patients with or without TNF- α for 48 h. MH7A cells respond to stimulation by nuclear factor kappa B (NF- κ B) activator, TNF- α , and IL-1 β to secrete cytokines such as IL-6 [31, 48]. MH7A responsiveness was quantified by measuring the IL-6 concentration of the MH7A-conditioned media. We found that the IL-6 secretion of MH7A significantly increases by TNF- α stimulation: TNF- α (+), 32.9 ± 2.4 pg/1000 cells; TNF- α (–), 0.245 ± 0.031 pg/1000 cells (see Fig 3). The results demonstrated that MH7A cells exhibit inflammatory response in our experimental conditions.

Assessment of exosomes derived from MH7A cells

Samples were isolated from MH7A-conditioned media (MH7A cells were cultured with or without TNF- α stimulation). To detect exosomes in the isolated samples, we analyzed exosomal marker proteins (CD63, CD81, and HSP70 with Western blot analysis (Fig 4A). We found CD63 enrichment and detected CD81 in the samples. However, we did not find HSP70. In total cellular lysates, CD63 and HSP70 production was observed. Previous studies have shown that exosomal markers, including HSP70, are produced variably in exosomes derived from various cells [49]. This variability explains the nondetectable levels of HSP70 in MH7A exosomes. RNAs extracted from exosomes with synthesized carrier RNA (MS2 bacteriophage RNA) were analyzed with an Agilent Bioanalyzer RNA 6000 Pico chip. We found no rRNA peaks (two distinct peaks in the >1500 nt region), and cellular RNAs were not contaminated in the exosome samples with or without TNF- α stimulation (Fig 4B). In this study, we added

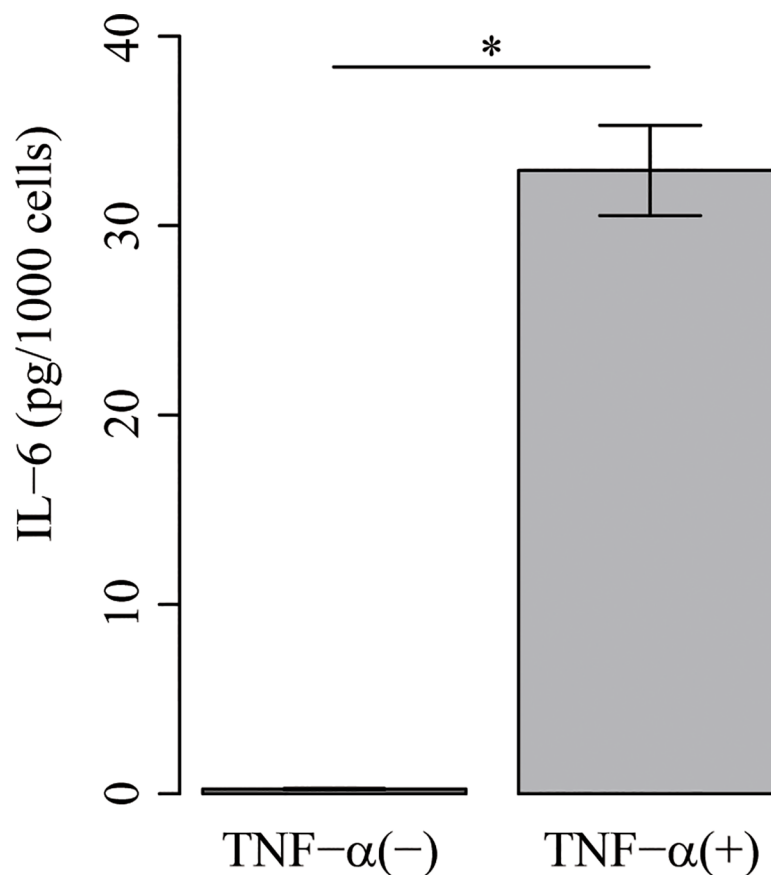


Fig 3. IL-6 secretion with or without TNF- α stimulation. Values are given as mean \pm standard error ($n = 3$).
*Statistical significance was determined by Welch's t-test ($P < 0.01$).

<https://doi.org/10.1371/journal.pone.0201851.g003>

carrier RNA to extract very small amount of exosomal RNAs. The amount of carrier RNA account for the majority, therefore, the peak profiles were very similar to those of the sample containing only carrier RNA.

Sequencing of exosomal and cellular RNAs derived from MH7A cells

The small RNA-Seq of exosomal and cellular RNAs of MH7A cells was performed with Illumina MiSeq. Spiked-in carrier RNAs were sequenced, along with exosomal RNAs; thus, we eliminated the sequence reads of carrier RNAs from exosome sample libraries by mapping to the carrier RNA sequence (5' -UCGCGACGUAUCGUGAUAUGGUUUUACAUAACGAUGCAC-3') by using Bowtie (version 0.12.7). The adaptor-ligated carrier RNA library was sequenced as a control. Exosomal and cellular RNAs with or without TNF- α stimulation were sequenced three times independently; thus, we sequenced 13 sequencing libraries and generated 66,043,334 reads (S1 Table). After preprocessing and eliminating carrier RNA sequence reads for exosome libraries, we found that 1,565,748 and 19,843,207 reads were aligned to the human genome in exosome libraries and cell libraries, respectively (S1 Table). Diverse categories of RNAs annotated by Ensembl were detected similar to those in exosomal RNA libraries, whereas miRNAs comprised a large proportion in cellular RNA samples (see Fig 5 and S1 File). Table 1 shows the expressions of transcripts belonging to RNAs other than miRNAs. Among these, the most abundant transcript was one of Y RNAs, RNY4 [19], which was not significantly induced with TNF- α stimulation (Table 1).

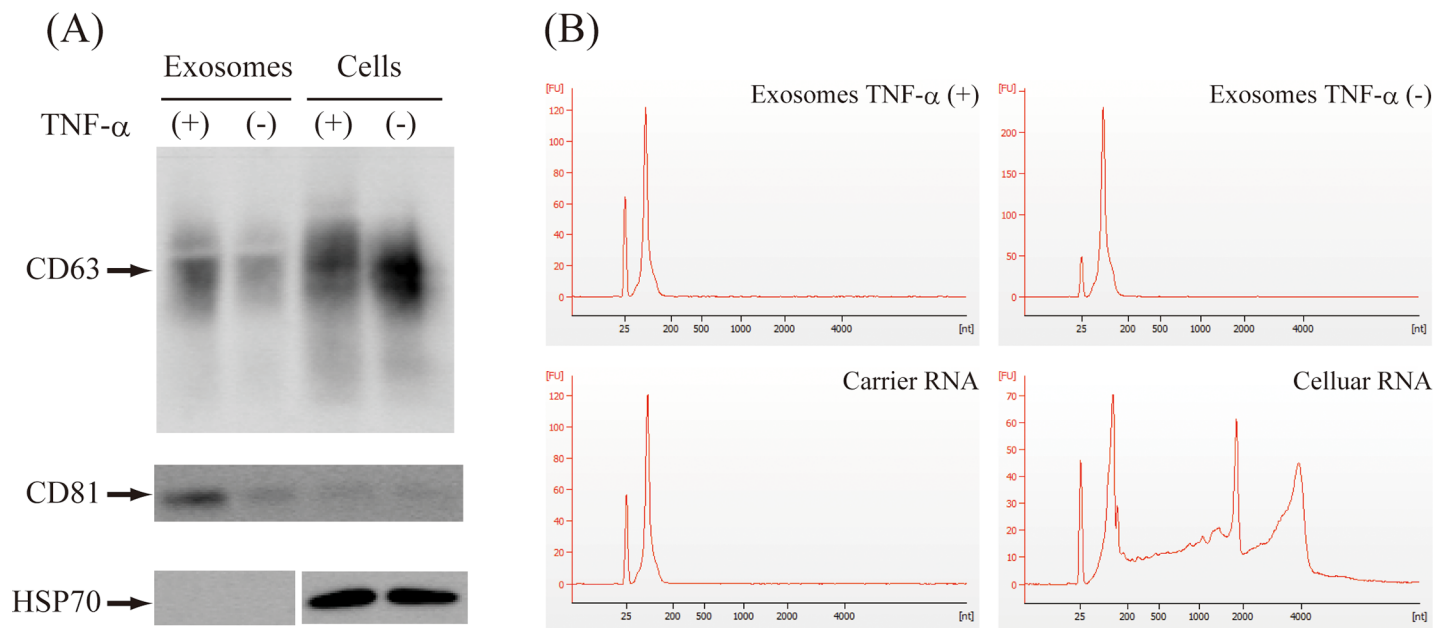


Fig 4. Assessment of exosomes derived from MH7A cells. (A) The Western blot analysis of exosome markers. (B) The size distribution of RNA molecules extracted from exosomes with or without TNF- α stimulation. Carrier RNA was also analyzed as a control. The cellular RNAs of MH7A were loaded as a positive control for rRNAs. The vertical axis shows fluorescent units (FU), and the scales were individually set. The horizontal axis shows electrophoretic mobility (RNA length). Lower marker peaks appeared at 25 nt. FU, fluorescent units. nt, nucleotides.

<https://doi.org/10.1371/journal.pone.0201851.g004>

The number of reads mapped to the mature miRNA sequences in miRBase (release 21) were 485,507 and 12,747,590 in exosome libraries and cell libraries, respectively (S1 Table and S2 File). On average, we detected 223 and 663 known miRNAs with ≥ 5 coverage in exosome libraries and cell libraries, respectively. In the adaptor-ligated carrier RNA library, only 340 reads were aligned to the mature miRNA sequence in miRBase (release 21) (S1 Table and S2 File). Among these 340 reads, most were aligned to miR-21-5p (122 reads) and miR-100-5p (41 reads), which were abundant in other libraries (S2 Table and S2 File). This may be explained either by cross-contamination between samples during library precipitation or by the false assignment of index sequences to the samples in Illumina MiSeq sequencing (also known as index hopping) [50]. The number of miRNA reads detected in the adaptor-ligated carrier RNA library was extremely small compared with that of other libraries; hence, we concluded that the use of carrier RNA would not affect exosomal RNA-Seq.

Among the exosomal miRNAs detected, four miRNAs (miR-155-5p, miR-146a-5p, miR-323a-5p, and miR-1307-3p) were significantly upregulated by TNF- α stimulation (FDR < 0.05) (red dots in Fig 6A, Table 2, and S3 File); significantly downregulated miRNAs were not detected in the exosomes. Among the cellular miRNAs detected, three miRNAs (miR-146a-5p, miR-184, and miR-146a-3p) were differentially expressed by TNF- α stimulation (FDR < 0.05) (red dots in Fig 6B, Table 2, and S3 File). miR-146a-5p and miR-146a-3p were significantly upregulated, whereas miR-184 was downregulated. By comparing the expression changes between exosomes and cells, we found that the expression variations of miR-323a-5p and miR-1307-3p were less than two times in the cells even though miR-155-5p and miR-146a-5p expressions were upregulated more than twice in both exosomes and cells (Table 3). These observations suggested that MH7A cells with TNF- α stimulation may preferentially load miR-323a-5p and miR-1307-3p onto exosomes (Table 3).

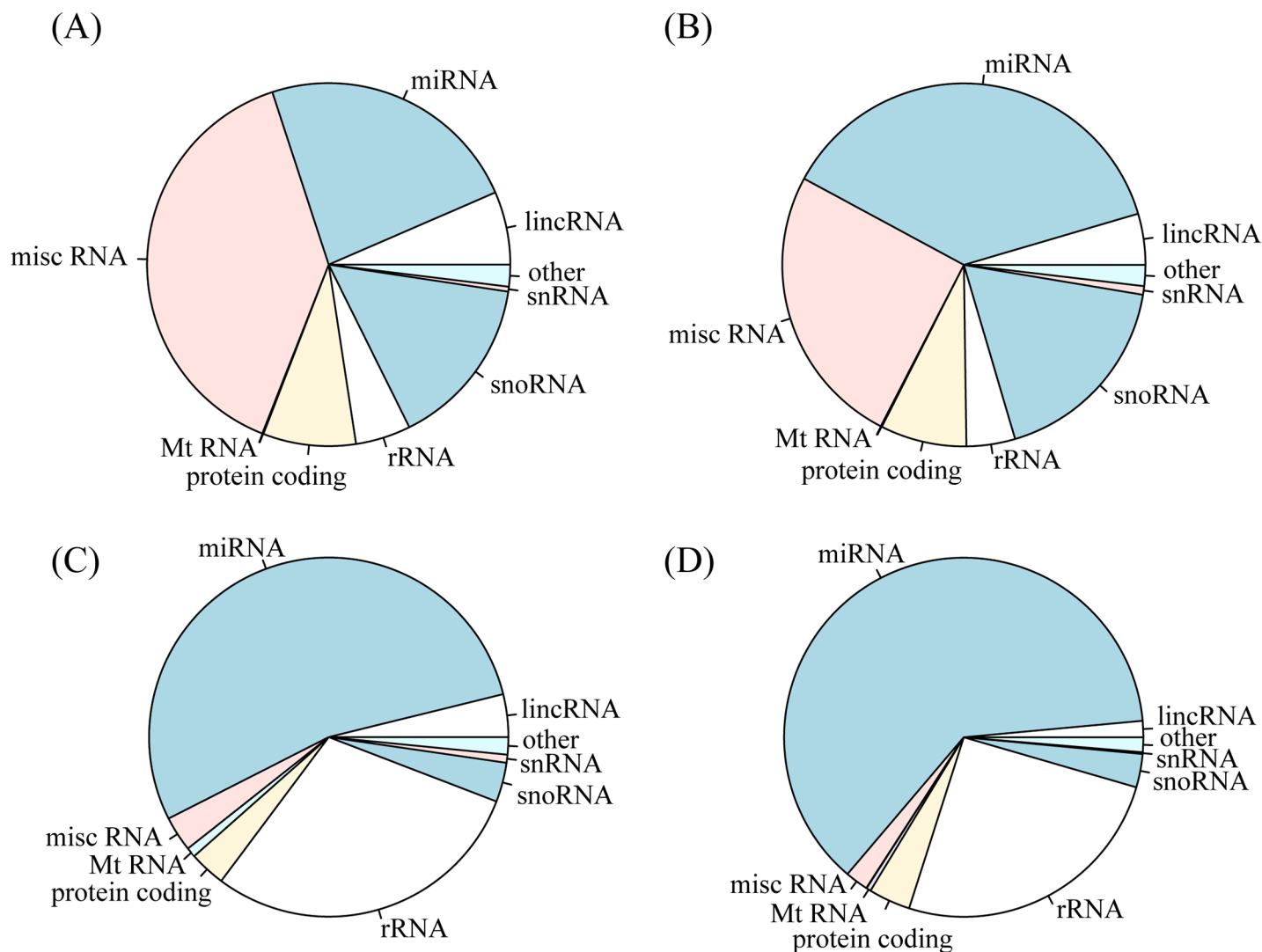


Fig 5. Distribution of RNA species. Exosomal RNAs with (A) or without (B) TNF- α stimulation. Cellular RNAs with (C) or without (D) TNF- α stimulation. lincRNA, long intergenic noncoding RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; mtRNA, mitochondrial RNA; misc RNA, miscellaneous RNA.

<https://doi.org/10.1371/journal.pone.0201851.g005>

Among the four miRNAs differentially expressed in exosomes, miR-323a-5p and miR-1307-3p were first annotated as possible candidates for RA-related miRNAs. By using DIANA-microT-CDS and TargetScan, we obtained 336 and 20 predicted target genes of miR-323a-5p and miR-1307-3p, respectively (S3 Table). The miR-323a-5p-predicted target genes included CD6 which is produced on the T-cell plasma membrane [51], whereas the miR-1307-3p-predicted target genes included *NDRG2* which reportedly affects osteoclast differentiation [52]. These predicted genes may be involved in RA pathogenesis.

Discussion

In this study, we quantified RASF exosomal RNAs with TNF- α stimulation. This is the first study to comprehensively analyze RASF-derived exosomal miRNAs. We found that four miRNAs (miR-155-5p, miR-146a-5p, miR-323a-5p, and miR-1307-3p) were upregulated in RASF exosomes by TNF- α stimulation (as shown in Fig 6A and Table 2). The selective loading of

Table 1. Top 10 most abundant transcripts other than miRNAs in exosomes.

Gene name	Ensemble ID	Gene type	Log2 FC	Log2 CPM	P Value	FDR
RNY4	ENSG00000252316.1	misc RNA	0.857	16.6	0.552	0.9
RNY4P10	ENSG00000202441.2	misc RNA	0.856	16.6	0.552	0.9
RNY4P7	ENSG00000201470.1	misc RNA	0.858	16.5	0.548	0.9
RNY4P20	ENSG00000252487.1	misc RNA	0.847	16.5	0.546	0.9
SNORD3A	ENSG00000263934.4	snoRNA	-0.0875	15.8	0.943	0.987
SNORD3C	ENSG00000264940.4	snoRNA	-0.0878	15.8	0.942	0.987
SNORD3B-2	ENSG00000262074.6	snoRNA	-0.0895	15.8	0.941	0.987
SNORD3B-1	ENSG00000265185.5	snoRNA	-0.0895	15.8	0.941	0.987
RNY1	ENSG00000201098.1	misc RNA	0.467	15.8	0.725	0.947
VTRNA1-1	ENSG00000199990.1	misc RNA	0.254	14.2	0.0654	0.798

FC, fold change; CPM, counts per million; FDR, false discovery rate; misc RNA, miscellaneous RNA; snoRNA, small nucleolar RNA.

<https://doi.org/10.1371/journal.pone.0201851.t001>

miRNA into exosomes have been reported [13, 53, 54]. For example, nSMase2 regulated the number of exosomal miRNAs [55]. Sumoylated hnRNPA2B1 recognizes 3' motif of RNAs and selectively packed them into exosomes [56]. Furthermore AGO2 was identified in exosomal proteins [57], and knockout of AGO2 could decrease the preferentially-loaded miRNAs [58]. However, there are still ambiguity in TNF- α -dependent or-independent mechanisms, and they remain to be clarified.

Our sequencing data indicated that MH7A exosomes contain various types of small RNAs annotated in Ensembl, such as snoRNAs, lincRNA, and misc RNA, as well as miRNAs. This

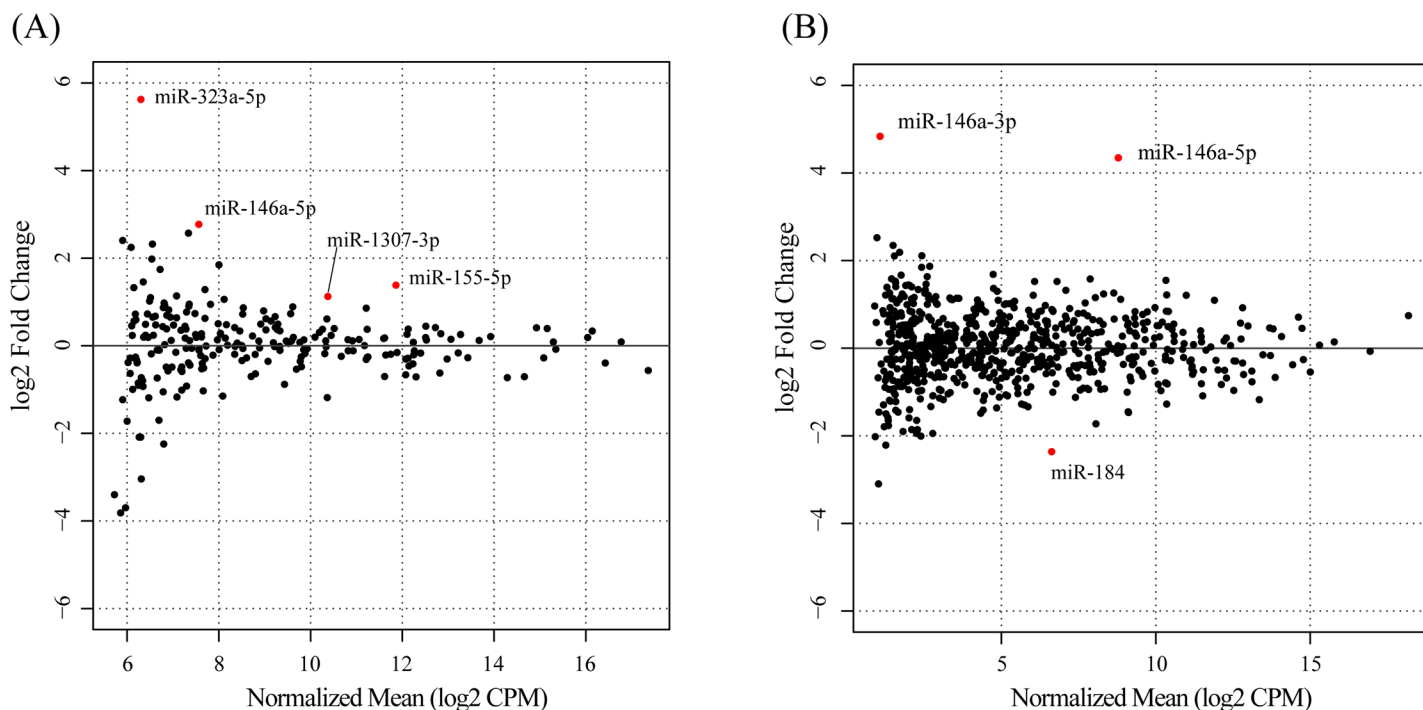


Fig 6. MA plot (stimulated vs. nonstimulated) of miRNAs. Exosomal RNAs (A) and cellular RNAs (B). Each point represents one miRNA. miRNAs upregulated with TNF- α stimulation are above the center horizontal line, and miRNAs downregulated with TNF- α stimulation are below the center horizontal line. miRNAs with FDR < 0.05 are shown as red dots. CPM, counts per million.

<https://doi.org/10.1371/journal.pone.0201851.g006>

Table 2. Differentially expressed miRNAs.

	miRNA	Log ₂ FC	Log ₂ CPM	P Value	FDR
Exosomes	miR-155-5p	1.38	11.9	0.0000191	0.00456
	miR-146a-5p	2.77	7.56	0.000145	0.0174
	miR-323a-5p	5.62	6.30	0.000388	0.0268
	miR-1307-3p	1.13	10.4	0.000448	0.0268
Cells	miR-146a-5p	3.57	9.41	4.70×10^{-14}	3.34×10^{-11}
	miR-184	-2.36	6.63	8.55×10^{-6}	0.00304
	miR-146a-3p	4.02	2.17	6.71×10^{-5}	0.0159

FC, fold change; CPM, counts per million; FDR, false discovery rate.

<https://doi.org/10.1371/journal.pone.0201851.t002>

result is consistent with previous reports that exosomes contain various types of RNAs [59]. In particular, we found that misc RNAs are conspicuously expressed in exosomes, along with miRNAs. In the misc RNAs identified in our study, Y RNAs were included. This is consistent with previous studies showing the existence of Y RNAs in exosomes [59]. hY4 (gene names annotated as RNY4), one of the Y RNAs abundantly present in MH7A exosomes (shown in Table 1), is suggested as a signaling driver of Toll-like receptor 7 in monocytes [29]. Therefore, although hY4 was not differentially expressed with TNF- α stimulation in the present study (Table 1), abundant exosomal hY4 might contribute to an inflammatory positive-feedback loop in RA-affected joints. However, further studies are required to clarify the function of extracellular Y RNAs and the loading mechanism of these RNAs.

It is noteworthy that miR-155-5p and miR-146a-5p, which are reportedly involved in RA pathogenesis [60], were expressed in exosomes derived from the RASF cell line MH7A and upregulated with TNF- α stimulation. A previous study reported that miR-155-5p and miR-146a-5p are upregulated in the synovial fluid in RA compared with osteoarthritis (OA) [22]. This suggested that our experimental model is valid. With RASF-aberrant growth and abundance in the synovium in RA [2], upregulated miR-155-5p and miR-146a-5p in RASF exosomes appear to contribute to an increase in these miRNAs in the synovial fluid in RA.

miR-155-5p is reportedly involved in RA pathogenesis and highly expressed in synovial fibroblasts [23, 61], synovial tissues [23], peripheral blood mononuclear cells (PBMCs) [61], and macrophages [62] in RA patients compared with healthy controls or OA patients. miR-155-5p also regulates inflammation response and the development of each lymphocyte subset, such as B-cells [63], CD8+ [64], and CD4+ T-cells [65]. miR-155-deficient mice do not develop collagen-induced arthritis (an RA model in mice); hence, miR-155 has been implicated as a crucial miRNA in RA pathogenesis [24]. On the other hand, miR-155-5p is confirmed to suppress production of matrix metalloproteinase-3 production via the downregulation of the inhibitor of NF- κ B kinase subunit epsilon, thus suggesting that miR-155-5p is a protective

Table 3. Comparison of expression variation of four miRNAs significantly upregulated in exosomes.

miRNA	Log ₂ FC in cells	Log ₂ FC in exosomes
miR-155-5p	1.09	1.38
miR-146a-5p	3.56	2.77
miR-323a-5p	0.261	5.62
miR-1307-3p	-0.269	1.13

FC, fold change.

<https://doi.org/10.1371/journal.pone.0201851.t003>

factor in joint destruction [61]. Therefore, the intercellular transportation of miR-155-5p derived from RASF exosomes might play a dual role in the development of arthritis and protection from joint destruction.

The roles of miR-146a-5p in RA seem confusing [21, 60]. miR-146a-5p expression is reportedly upregulated by TNF- α stimulation in vitro [23, 66] and increases in synovial tissue [66], synovial fibroblasts [23], CD4⁺ T-cells [67], PBMCs [68], synovial fluid [22], and plasma [22] in RA patients compared with healthy controls. Studies have confirmed that miR-146a-5p targets TNF receptor associated factor 6 (TRAF-6) and interleukin-1 receptor associated kinase 1 (IRAK-1) in vitro [69]. However, despite increased miR-146a-5p expression, TRAF-6 and IRAK-1 production did not change significantly in PBMCs obtained from RA patients compared with healthy controls [68]. Furthermore, miR-146a-5p overexpression suppressed apoptosis in Jurkat cells used as a T-cell model, thus indicating that miR-146-5p is an inflammatory agent [67]. On the other hand, the administration of double-stranded miR-146a-5p prevented joint destruction in a collagen-induced arthritis mouse model [70]. Considering that miR-146a-5p can be a positive or negative regulator of inflammation and joint destruction, our observation that miR-146a-5p is detected and upregulated with TNF- α stimulation in RASF exosomes would help us determine the role of miR-146a-5p in RA pathogenesis.

We observed that miR-323a-5p and miR-1307-3p, as well as miR-155-5p and miR-146a-5p, were upregulated with TNF- α stimulation in exosomes. miR-323a-5p and miR-1307-3p were first shown to be relevant in RA, and further studies are required to detect these miRNAs *in vivo*. Among the predicted target genes, we included those whose functions are relevant to inflammation and joint destruction: miR-323a-5p can target CD6, and miR-1307-3p can target *NDRG2* (S3 Table). CD6 is an outer membrane protein of T-cells [71]. It was formerly regarded a costimulatory receptor of T-lymphocytes [72, 73] but has been revealed to attenuate the signaling of T-cell activation [74]. Therefore, miR-323a-5p transport to T-cells has the potential to reinforce synovium inflammation in RA. The overexpression of *NDRG2*, one of the predicted target genes of miR-1307-3p, has been shown to inhibit osteoclast differentiation from monocytes [52]. Furthermore, *NDRG2* inhibits the expression of osteoclast-related genes, including the receptor activator of NF- κ B (RANK) in monocytes [52]. By contributing to osteoclastogenesis [75] in a coordinated manner with RANK ligand expression, the exosomal miR-1307-3p transported to monocytes has the potential to promote osteoclast differentiation by targeting *NDRG2* and cause joint destruction.

We found that in our sequencing data, exosomal and intracellular miRNA expression with TNF- α stimulation was different among miR-155-5p, miR-146a-5p, miR-323a-5p, and miR-1307-3p. miR-146a-5p was significantly upregulated in both exosomes and cells, and miR-155-5p was upregulated by twofold in both. By contrast, miR-323a-5p and miR-1307-3p were significantly upregulated only in exosomes, and the intracellular expression variation between them was less than twofold. Studies have reported that a subset of miRNAs is preferentially sorted from the cytosol to exosomes [27, 76–78]. Thus, MH7A cells with TNF- α stimulation may preferentially load miR-323a-5p and miR-1307-3p to exosomes.

The role of exosomes in RA pathogenesis has been investigated earlier but remains unclear [79]. Exosomes contain numerous bioactive molecules, such as proteins, mRNAs, miRNAs, and other RNAs. The physiological activity of exosomes reflects the multiple effects of their molecular cargo. In the case of RASF exosomes, T-cell resistance to apoptosis is neutralized only partly by soluble TNF receptor-1, thus indicating that exosomal molecular cargo other than TNF- α contributes to T-cell apoptosis resistance [17]. Furthermore, the physiological activity of exosomes differs among recipient cell types. Our findings on RASF exosomal RNAs suggest that future studies using more physiological models are required to identify target cells and to explore the physiological activity of RASF exosomes.

In conclusion, our study quantified small RNAs in RASF exosomes. Differential expression analysis identified that four miRNAs are differentially expressed in exosomes with TNF- α stimulation. Our data on the RNA molecular cargo of RASF exosomes will help us in understanding the role of exosomes in RA pathogenesis.

Supporting information

S1 Table. Summary of 13 libraries.

(XLSX)

S2 Table. Top 10 abundant miRNAs in exosome and cell. CPM, counts per million. FC, fold change.

(XLSX)

S3 Table. Predicted target genes of miR-1307-3p and miR-323a-5p. Prediction score threshold was set to 0.7 as default.

(XLSX)

S1 File. Transcripts read counts of all libraries.

(XLSX)

S2 File. miRNA read counts of all libraries.

(XLSX)

S3 File. TMM normalization and differential expression of miRNAs. CPM, counts per million. FC, fold change. FDR, false discovery rate.

(XLSX)

Acknowledgments

This work was supported by JST CREST (Grant No. JPMJCR16G2).

Author Contributions

Conceptualization: Yosuke Takamura, Atsushi Satomura, Mitsuyoshi Ueda.

Data curation: Yosuke Takamura, Wataru Aoki.

Formal analysis: Yosuke Takamura.

Funding acquisition: Mitsuyoshi Ueda.

Investigation: Yosuke Takamura.

Methodology: Yosuke Takamura, Wataru Aoki, Mitsuyoshi Ueda.

Project administration: Mitsuyoshi Ueda.

Resources: Seiji Shibasaki.

Software: Atsushi Satomura.

Supervision: Wataru Aoki, Atsushi Satomura, Mitsuyoshi Ueda.

Validation: Wataru Aoki, Mitsuyoshi Ueda.

Writing – original draft: Yosuke Takamura, Wataru Aoki, Atsushi Satomura, Mitsuyoshi Ueda.

Writing – review & editing: Yosuke Takamura, Wataru Aoki, Mitsuyoshi Ueda.

References

1. Scott DL, Wolfe F, Huizinga TWJ. Rheumatoid arthritis. *The Lancet*. 2010 Sep 25; 376(9746):1094–108. [https://doi.org/10.1016/S0140-6736\(10\)60826-4](https://doi.org/10.1016/S0140-6736(10)60826-4)
2. Turner JD, Filer A. The role of the synovial fibroblast in rheumatoid arthritis pathogenesis. *Curr Opin Rheumatol*. 2015 Mar; 27(2):175–82. <https://doi.org/10.1097/BOR.0000000000000148> PMID: 25603041
3. McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. *New England Journal of Medicine*. 2011; 365(23):2205–19. <https://doi.org/10.1056/NEJMra1004965> PMID: 22150039
4. Shibasaki S, Karasaki M, Aburaya S, Morisaka H, Takeda Y, Aoki W, et al. A comparative proteomics study of a synovial cell line stimulated with TNF- α . *FEBS Open Bio*. 2016; 6(5):418–24. <https://doi.org/10.1002/2211-5463.12049> PMID: 27419047
5. Davis JM III, Matteson EL. My Treatment Approach to Rheumatoid Arthritis. *Mayo Clinic Proceedings*. 2012; 87(7):659–73. <https://doi.org/10.1016/j.mayocp.2012.03.011> PMID: 22766086
6. Maas SLN, Breakefield XO, Weaver AM. Extracellular Vesicles: Unique Intercellular Delivery Vehicles. *Trends in Cell Biology*. 2017; 27(3):172–88. <https://doi.org/10.1016/j.tcb.2016.11.003> PMID: 27979573
7. Yáñez-Mó M, Siljander PRM, Andreu Z, Bedina Zavec A, Borràs FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. *Journal of Extracellular Vesicles*. 2015 Jan 01; 4(1):27066. <https://doi.org/10.3402/jev.v4.27066> PMID: 25979354
8. Buzas EI, Gyorgy B, Nagy G, Falus A, Gay S. Emerging role of extracellular vesicles in inflammatory diseases. *Nat Rev Rheumatol*. 2014 06/print; 10(6):356–64. <https://doi.org/10.1038/nrrheum.2014.19> PMID: 24535546
9. Kalluri R. The biology and function of exosomes in cancer. *The Journal of clinical investigation*. 2016 Apr 1; 126(4):1208–15. <https://doi.org/10.1172/JCI81135> PMID: 27035812
10. Tominaga N, Kosaka N, Ono M, Katsuda T, Yoshioka Y, Tamura K, et al. Brain metastatic cancer cells release microRNA-181c-containing extracellular vesicles capable of destructing blood-brain barrier. *Nature communications*. 2015 Apr 1; 6:6716. <https://doi.org/10.1038/ncomms7716> PMID: 25828099
11. He C, Zheng S, Luo Y, Wang B. Exosome Theranostics: Biology and Translational Medicine. *Theranostics*. 2018; 8(1):237–55. <https://doi.org/10.7150/thno.21945> PMID: 29290805
12. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nature reviews Molecular cell biology*. 2018 Apr; 19(4):213–28. <https://doi.org/10.1038/nrm.2017.125> PMID: 29339798
13. Zhang J, Li S, Li L, Li M, Guo C, Yao J, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics, proteomics & bioinformatics*. 2015 Feb; 13(1):17–24. <https://doi.org/10.1016/j.gpb.2015.02.001> PMID: 25724326
14. Makarova JA, Shkurnikov MU, Wicklein D, Lange T, Samatov TR, Turchinovich AA, et al. Intracellular and extracellular microRNA: An update on localization and biological role. *Progress in histochemistry and cytochemistry*. 2016 Nov; 51(3–4):33–49. <https://doi.org/10.1016/j.proghi.2016.06.001> PMID: 27396686
15. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proceedings of the National Academy of Sciences of the United States of America*. 2012 Jul 31; 109(31):E2110–6. <https://doi.org/10.1073/pnas.1209414109> PMID: 22753494
16. Skriner K, Adolph K, Jungblut PR, Burmester GR. Association of citrullinated proteins with synovial exosomes. *Arthritis & Rheumatism*. 2006; 54(12):3809–14. <https://doi.org/10.1002/art.22276> PMID: 17133577
17. Zhang HG, Liu C, Su K, Yu S, Zhang L, Zhang S, et al. A membrane form of TNF- α presented by exosomes delays T cell activation-induced cell death. *J Immunol*. 2006 Jun 15; 176(12):7385–93. PMID: 16751383
18. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. *Nucleic acids research*. 2014 Jan 1; 42(D1):D68–D73. <https://doi.org/10.1093/nar/gkt1181> PMID: 24275495
19. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nature reviews Molecular cell biology*. 2005 May; 6(5):376–85. <https://doi.org/10.1038/nrm1644> PMID: 15852042
20. Mehta A, Baltimore D. MicroRNAs as regulatory elements in immune system logic. *Nature Reviews Immunology*. 2016 Apr 28; 16:279. <https://doi.org/10.1038/nri.2016.40> PMID: 27121651
21. Salehi E, Eftekhari R, Oraei M, Gharib A, Bidad K. MicroRNAs in rheumatoid arthritis. *Clin Rheumatol*. 2015 Apr; 34(4):615–28. <https://doi.org/10.1007/s10067-015-2898-x> PMID: 25736037

22. Murata K, Yoshitomi H, Tanida S, Ishikawa M, Nishitani K, Ito H, et al. Plasma and synovial fluid micro-RNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis. *Arthritis Research & Therapy*. 2010; 12(3):86–8.
23. Stanczyk J, Pedrioli DML, Brentano F, Sanchez-Pernaute O, Kolling C, Gay RE, et al. Altered expression of MicroRNA in synovial fibroblasts and synovial tissue in rheumatoid arthritis. *Arthritis & Rheumatism*. 2008; 58(4):1001–9. <https://doi.org/10.1002/art.23386> PMID: 18383392
24. Blüml S, Bonelli M, Niederreiter B, Puchner A, Mayr G, Hayer S, et al. Essential role of microRNA-155 in the pathogenesis of autoimmune arthritis in mice. *Arthritis & Rheumatism*. 2011; 63(5):1281–8. <https://doi.org/10.1002/art.30281> PMID: 21321928
25. Patil VS, Zhou R, Rana TM. Gene regulation by noncoding RNAs. *Critical reviews in biochemistry and molecular biology*. 2014; 49(1):16–32. <https://doi.org/10.3109/10409238.2013.844092> PMID: 24164576
26. Kowalski MP, Krude T. Functional roles of non-coding Y RNAs. *The International Journal of Biochemistry & Cell Biology*. 2015 Sep 1; 66:20–9. <https://doi.org/10.1016/j.biocel.2015.07.003> PMID: 26159929
27. Nolte-’t Hoen ENM, Buermans HPJ, Waasdorp M, Stoorvogel W, Wauben MHM, ’t Hoen PAC. Deep sequencing of RNA from immune cell-derived vesicles uncovers the selective incorporation of small non-coding RNA biotypes with potential regulatory functions. *Nucleic acids research*. 2012; 40(18):9272–85. <https://doi.org/10.1093/nar/gks658> PMID: 22821563
28. Vojtech L, Woo S, Hughes S, Levy C, Ballweber L, Sauteraud RP, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic acids research*. 2014; 42(11):7290–304. <https://doi.org/10.1093/nar/gku347> PMID: 24838567
29. Haderk F, Schulz R, Iskar M, Cid LL, Worst T, Willmund KV, et al. Tumor-derived exosomes modulate PD-L1 expression in monocytes. *Science Immunology*. 2017; 2(13). <https://doi.org/10.1126/sciimmunol.aah5509> PMID: 28754746
30. Baglio S, Rooijers K, Koppers-Lalic D, Verweij F, Pérez Lanzón M, Zini N, et al. Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Research & Therapy*. 2015 Jul 1; 6(1):1–20. <https://doi.org/10.1186/s13287-015-0116-z> PMID: 26129847
31. Miyazawa K, Mori A, Okudaira H. Establishment and Characterization of a Novel Human Rheumatoid Fibroblast-Like Synoviocyte Line, MH7A, Immortalized with SV40 T Antigen. *Journal of Biochemistry*. 1998 December 1, 1998; 124(6):1153–62. <https://doi.org/10.1093/oxfordjournals.jbchem.a022233> PMID: 9832620
32. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*. 2012 Jun 28; 9:671. <https://doi.org/10.1038/nmeth.2089> PMID: 22930834
33. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol*. 2006 Apr;Chapter 3:Unit 3 22. <https://doi.org/10.1002/0471143030.cb0322s30> PMID: 18228490
34. Hill AF, Pegtel DM, Lambertz U, Leonardi T, O’Driscoll L, Pluchino S, et al. ISEV position paper: extracellular vesicle RNA analysis and bioinformatics. *Journal of Extracellular Vesicles*. 2013; 2. <https://doi.org/10.3402/jev.v2i0.22859> PMID: 24376909
35. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic acids research*. 1997; 25(17):3389–402. <https://doi.org/10.1093/nar/25.17.3389> PMID: 9254694
36. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet Journal*. 2011; 17(1):pp. 10–2.
37. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biology*. 2009; 10(3):1–10. <https://doi.org/10.1186/gb-2009-10-3-r25> PMID: 19261174
38. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009; 25(16):2078–9. <https://doi.org/10.1093/bioinformatics/btp352> PMID: 19505943
39. Sun Z, Evans J, Bhagwate A, Middha S, Bockol M, Yan H, et al. CAP-miRSeq: a comprehensive analysis pipeline for microRNA sequencing data. *BMC genomics*. 2014; 15(1):1–10. <https://doi.org/10.1186/1471-2164-15-423> PMID: 24894665
40. Zerbino DR, Achuthan P, Akanni W, Amode M R, Barrell D, Bhai J, et al. Ensembl 2018. *Nucleic acids research*. 2018; 46(D1):D754–D61. <https://doi.org/10.1093/nar/gkx1098> PMID: 29155950
41. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. *Genome Biology*. 2010 Mar 2; 11(3):R25. <https://doi.org/10.1186/gb-2010-11-3-r25> PMID: 20196867

42. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic acids research*. 2012; 40(10):4288–97. <https://doi.org/10.1093/nar/gks042> PMID: 22287627
43. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics*. 2010; 26(1):139–40. <https://doi.org/10.1093/bioinformatics/btp616> PMID: 19910308
44. Robinson MD, Smyth GK. Small-sample estimation of negative binomial dispersion, with applications to SAGE data. *Biostatistics*. 2008; 9(2):321–32. <https://doi.org/10.1093/biostatistics/kxm030> PMID: 17728317
45. Paraskevopoulou MD, Georgakilas G, Kostoulas N, Vlachos IS, Vergoulis T, Reczko M, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. *Nucleic acids research*. 2013 May 16; 41(Web Server issue):W169–W73. <https://doi.org/10.1093/nar/gkt393> PMID: 23680784
46. Reczko M, Maragkakis M, Alexiou P, Grosse I, Hatzigeorgiou AG. Functional microRNA targets in protein coding sequences. *Bioinformatics*. 2012; 28(6):771–6. <https://doi.org/10.1093/bioinformatics/bts043> PMID: 22285563
47. Lewis BP, Burge CB, Bartel DP. Conserved Seed Pairing, Often Flanked by Adenosines, Indicates that Thousands of Human Genes are MicroRNA Targets. *Cell*. 2005 Jan 14; 120(1):15–20. <https://doi.org/10.1016/j.cell.2004.12.035> PMID: 15652477
48. Li J, Li J, Yue Y, Hu Y, Cheng W, Liu R, et al. Genistein suppresses tumor necrosis factor α -induced inflammation via modulating reactive oxygen species/Akt/nuclear factor κ B and adenosine monophosphate-activated protein kinase signal pathways in human synovial cell MH7A cells. *Drug Design, Development and Therapy*. 2014 Mar 17; 8:315–23. <https://doi.org/10.2147/DDDT.S52354> PMID: 24669186
49. Yoshioka Y, Konishi Y, Kosaka N, Katsuda T, Kato T, Ochiya T. Comparative marker analysis of extracellular vesicles in different human cancer types. *Journal of extracellular vesicles*. 2013; 2: <https://doi.org/10.3402/jev.v2i0.20424> PMID: 24009892
50. Kircher M, Sawyer S, Meyer M. Double indexing overcomes inaccuracies in multiplex sequencing on the Illumina platform. *Nucleic acids research*. 2012 Oct 21; 40(1):e3–e. <https://doi.org/10.1093/nar/gkr771> PMID: 22021376
51. Pinto M, Carmo AM. CD6 as a Therapeutic Target in Autoimmune Diseases: Successes and Challenges. *BioDrugs*. 2013 Jun 1; 27(3):191–202. <https://doi.org/10.1007/s40259-013-0027-4> PMID: 23568178
52. Kang K, Nam S, Kim B, Lim JH, Yang Y, Lee M-S, et al. Inhibition of osteoclast differentiation by overexpression of NDRG2 in monocytes. *Biochemical and Biophysical Research Communications*. 2015 Dec 25; 468(4):611–6. <https://doi.org/10.1016/j.bbrc.2015.10.167> PMID: 26546825
53. Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T. Secretory mechanisms and intercellular transfer of microRNAs in living cells. *The Journal of biological chemistry*. 2010 Jun 4; 285(23):17442–52. <https://doi.org/10.1074/jbc.M110.107821> PMID: 20353945
54. Pan BT, Johnstone R. Selective externalization of the transferrin receptor by sheep reticulocytes in vitro. Response to ligands and inhibitors of endocytosis. *The Journal of biological chemistry*. 1984 Aug 10; 259(15):9776–82. PMID: 6146620
55. Kosaka N, Iguchi H, Hagiwara K, Yoshioka Y, Takeshita F, Ochiya T. Neutral sphingomyelinase 2 (nSMase2)-dependent exosomal transfer of angiogenic microRNAs regulate cancer cell metastasis. *The Journal of biological chemistry*. 2013 Apr 12; 288(15):10849–59. <https://doi.org/10.1074/jbc.M112.446831> PMID: 23439645
56. Villarroya-Beltrí C, Gutierrez-Vazquez C, Sanchez-Cabo F, Perez-Hernandez D, Vazquez J, Martin-Cofreces N, et al. Sumoylated hnRNP A2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nature communications*. 2013; 4:2980. <https://doi.org/10.1038/ncomms3980> PMID: 24356509
57. Goldie BJ, Dun MD, Lin M, Smith ND, Verrills NM, Dayas CV, et al. Activity-associated miRNA are packaged in Map1b-enriched exosomes released from depolarized neurons. *Nucleic acids research*. 2014 Aug; 42(14):9195–208. <https://doi.org/10.1093/nar/gku594> PMID: 25053844
58. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC genomics*. 2012 Aug 1; 13:357. <https://doi.org/10.1186/1471-2164-13-357> PMID: 22849433
59. Cambier L, de Couto G, Ibrahim A, Echavez AK, Valle J, Liu W, et al. Y RNA fragment in extracellular vesicles confers cardioprotection via modulation of IL-10 expression and secretion. *EMBO Mol Med*. 2017 Feb 6; 9(3):337–52. <https://doi.org/10.15252/emmm.201606924> PMID: 28167565

60. Tavasolian F, Abdollahi E, Rezaei R, Momtazi-borojeni AA, Henrotin Y, Sahebkar A. Altered Expression of MicroRNAs in Rheumatoid Arthritis. *Journal of Cellular Biochemistry*. 2018; 119(1):478–87. <https://doi.org/10.1002/jcb.26205> PMID: 28598026
61. Long L, Yu P, Liu Y, Wang S, Li R, Shi J, et al. Upregulated MicroRNA-155 Expression in Peripheral Blood Mononuclear Cells and Fibroblast-Like Synoviocytes in Rheumatoid Arthritis. *Clinical and Developmental Immunology*. 2013 Sep 17; 2013:296139. <https://doi.org/10.1155/2013/296139> PMID: 24151514
62. Kurowska-Stolarska M, Alivernini S, Ballantine LE, Asquith DL, Millar NL, Gilchrist DS, et al. MicroRNA-155 as a proinflammatory regulator in clinical and experimental arthritis. *Proceedings of the National Academy of Sciences*. 2011; 108(27):11193–8. <https://doi.org/10.1073/pnas.1019536108> PMID: 21690378
63. Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al. microRNA-155 Regulates the Generation of Immunoglobulin Class-Switched Plasma Cells. *Immunity*. 2007 Dec 21; 27(6):847–59. <https://doi.org/10.1016/j.immuni.2007.10.009> PMID: 18055230
64. Lind EF, Elford AR, Ohashi PS. Micro-RNA 155 Is Required for Optimal CD8+ T Cell Responses to Acute Viral and Intracellular Bacterial Challenges. *The Journal of Immunology*. 2013; 190(3):1210–6. <https://doi.org/10.4049/jimmunol.1202700> PMID: 23275599
65. O'Connell RM, Kahn D, Gibson WSJ, Round JL, Scholz RL, Chaudhuri AA, et al. MicroRNA-155 Promotes Autoimmune Inflammation by Enhancing Inflammatory T Cell Development. *Immunity*. 2010 Oct 29; 33(4):607–19. <https://doi.org/10.1016/j.immuni.2010.09.009> PMID: 20888269
66. Nakasa T, Miyaki S, Okubo A, Hashimoto M, Nishida K, Ochi M, et al. Expression of microRNA-146 in rheumatoid arthritis synovial tissue. *Arthritis & Rheumatism*. 2008; 58(5):1284–92. <https://doi.org/10.1002/art.23429> PMID: 18438844
67. Li J, Wan Y, Guo Q, Zou L, Zhang J, Fang Y, et al. Altered microRNA expression profile with miR-146a upregulation in CD4+ T cells from patients with rheumatoid arthritis. *Arthritis Research & Therapy*. 2010; 12(3):1–12. <https://doi.org/10.1186/ar3006> PMID: 20459811
68. Pauley KM, Satoh M, Chan AL, Bubba MR, Reeves WH, Chan EKL. Upregulated miR-146a expression in peripheral blood mononuclear cells from rheumatoid arthritis patients. *Arthritis Research & Therapy*. 2008 Aug 29; 10(4):R101–R. <https://doi.org/10.1186/ar2493> PMID: 18759964
69. Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF- κ B-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proceedings of the National Academy of Sciences*. 2006; 103(33):12481–6. <https://doi.org/10.1073/pnas.0605298103> PMID: 16885212
70. Nakasa T, Shibuya H, Nagata Y, Niimoto T, Ochi M. The inhibitory effect of microRNA-146a expression on bone destruction in collagen-induced arthritis. *Arthritis & Rheumatism*. 2011; 63(6):1582–90. <https://doi.org/10.1002/art.30321> PMID: 21425254
71. Crescitelli R, Lasser C, Szabo TG, Kittel A, Eldh M, Dianzani I, et al. Distinct RNA profiles in subpopulations of extracellular vesicles: apoptotic bodies, microvesicles and exosomes. *Journal of Extracellular Vesicles*. 2013; 2: <https://doi.org/10.3402/jev.v2i0.20677> PMID: 24223256
72. Gangemi RM, Swack JA, Gaviria DM, Romain PL. Anti-T12, an anti-CD6 monoclonal antibody, can activate human T lymphocytes. *The Journal of Immunology*. 1989; 143(8):2439–47. PMID: 2794503
73. Osorio LM, Garcia CA, Jondal M, Chow SC. The Anti-CD6 mAb, IOR-T1, Defined a New Epitope on the Human CD6 Molecule That Induces Greater Responsiveness in T Cell Receptor/CD3-Mediated T Cell Proliferation. *Cellular Immunology*. 1994 Mar 1; 154(1):123–33. <https://doi.org/10.1006/cimm.1994.1062> PMID: 7509726
74. Oliveira MI, Gonçalves CM, Pinto M, Fabre S, Santos AM, Lee SF, et al. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. *European Journal of Immunology*. 2012 Sep 28; 42(1):195–205. <https://doi.org/10.1002/eji.201040528> PMID: 21956609
75. Shigeyama Y, Pap T, Kunzler P, Simmen BR, Gay RE, Gay S. Expression of osteoclast differentiation factor in rheumatoid arthritis. *Arthritis Rheum*. 2000 Nov; 43(11):2523–30. [https://doi.org/10.1002/1529-0131\(200011\)43:11<2523::AID-ANR20>3.0.CO;2-Z](https://doi.org/10.1002/1529-0131(200011)43:11<2523::AID-ANR20>3.0.CO;2-Z) PMID: 11083276
76. Guduric-Fuchs J, O'Connor A, Camp B, O'Neill CL, Medina RJ, Simpson DA. Selective extracellular vesicle-mediated export of an overlapping set of microRNAs from multiple cell types. *BMC genomics*. 2012 Aug 1; 13(1):357. <https://doi.org/10.1186/1471-2164-13-357> PMID: 22849433
77. Mittelbrunn M, Gutiérrez-Vázquez C, Villarroya-Beltrí C, González S, Sánchez-Cabo F, González MÁ, et al. Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nature communications*. 2011 Apr 19; 2:282. <https://doi.org/10.1038/ncomms1285> PMID: 21505438
78. Ohshima K, Inoue K, Fujiwara A, Hatakeyama K, Kanto K, Watanabe Y, et al. Let-7 MicroRNA Family Is Selectively Secreted into the Extracellular Environment via Exosomes in a Metastatic Gastric Cancer

Cell Line. PLOS ONE. 2010; 5(10):e13247. <https://doi.org/10.1371/journal.pone.0013247> PMID: [20949044](https://pubmed.ncbi.nlm.nih.gov/20949044/)

79. Withrow J, Murphy C, Liu Y, Hunter M, Fulzele S, Hamrick MW. Extracellular vesicles in the pathogenesis of rheumatoid arthritis and osteoarthritis. *Arthritis Research & Therapy*. 2016 Dec 1; 18:286. <https://doi.org/10.1186/s13075-016-1178-8> PMID: [27906035](https://pubmed.ncbi.nlm.nih.gov/27906035/)